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Blastomyces dermatitidis antigen detection in urine specimens from dogs with blastomycosis using a competitive binding inhibition ELISA

J.F. Shurley[1] A.M. Legendre[2] & G.M. Scalarone[1]
[1]*Department of Biological Sciences, Idaho State University, Pocatello, Idaho, 83209, USA;* [2]*University of Tennessee College of Veterinary Medicine, Knoxville, Tennessee, USA*

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Abstract

A competitive binding inhibition enzyme linked immunosorbent assay (ELISA) was used to detect *Blastomyces dermatitidis* antigens in urine specimens from dogs with blastomycosis. Sera from rabbits immunized with *B. dermatitidis* killed whole yeast cells were used as the primary antibody in the competitive ELISA. This initial study was performed to determine if *B. dermatitidis* antigen detection was possible and to test the efficacy of the rabbit sera as a primary antibody. An indirect ELISA was also performed to compare antigen detection in urine to antibody detection in the sera of the infected dogs. The results indicate 100% (36/36 specimens) detection of both antigen and antibody. Cross reactivity with *Histoplasma capsulatum*, as well as non-specific binding with the normal urine specimens, was observed with the competitive binding inhibition ELISA.

Key words: antigen detection, *Blastomyces dermatitidis*, blastomycosis, competitive ELISA, urine

Introduction

Blastomyces dermatitidis is a thermally dimorphic, systemic fungal organism affecting the deep tissues and organs of mammals, especially dogs and humans. The early symptoms in dogs can go unnoticed and detection may not occur until *B. dermatitidis* has disseminated [1, 2]. Humans with a compromised immune status due to organ transplants, chemotherapy, or AIDS may also be difficult to diagnose because the current immunodiagnostic techniques require production of an adequate antibody response. The techniques may lack the sensitivity to detect blastomycosis in compromised individuals. If the person cannot produce an adequate antibody response to *B. dermatitidis* the infection may go undetected. This may allow *B. dermatitidis* to disseminate systemically, before a diagnosis is made and antifungal therapy begins, causing greater illness in the patient.

The indirect ELISA is an important test for detection of antibodies against *B. dermatitidis* [3–8]. *Histoplasma capsulatum* is also a dimorphic fungal organism and is endemic in similar regions. Histoplasmosis infections were found to be falsely negative in 50% of the immunocompromised patients when using the indirect ELISA antibody detection technique [9]. However, the competitive ELISA technique, which detects antigens with the use of a known antibody, has successfully been used for the detection of histoplasmosis [10–13, 14–16]. The focus of this research was to determine if the competitive ELISA could be used to detect *B. dermatitidis* antigens using sera from rabbits immunized against *B. dermatitidis* as the primary antibody. Detecting antigens of *B. dermatitidis* instead of the antibody produced by the affected individual may help in establishing a diagnosis and may aid in monitoring the progress of anti-fungal therapy.

Materials and methods

Antigens

Yeast phase lysate antigens and whole cell antigens were prepared from various *B. dermatitidis* isolates. Mycelial phase cultures were converted to yeast cells by culturing on brain heart infusion agar with cysteine at 37 °C. The yeast phase reagents were prepared by a previously described method [17, 18] modified by Johnson and Scalarone [19]. Yeast cells were grown for 5 days at 37 °C with shaking in a chemically defined, nutritionally lean medium. The cells were harvested and washed five times by centrifugation for 5 min at $700 \times g$ in sterile distilled water. The cells were lysed by incubation in sterile water for 7 days at 37 °C with shaking. The suspension was centrifuged (30 min at $700 \times g$) to remove debris and filter sterilized through a 0.2 μm Nalgene filter (Nalge Company, Rochester, NY). Merthiolate (1:10,000) was added as a preservative to the lysate reagents. Protein determinations were made on the lysate antigen preparations using the bicinchoninic acid (BCA) method according to manufacturer directions (Sigma Chemical Company, St. Louis, MO). The whole yeast cells were prepared similarly as described in the rabbit immunization section. The lysate and whole cell reagents were stored at 4 °C for future use.

Rabbit immunization

Six female New Zealand white rabbits (2 kg) (Western Oregon Rabbit Company, Philometh, OR) were immunized intramuscularly and subcutaneously (1 ml per site) with a pool of killed *B. dermatitidis* whole yeast cells. The yeast cells were grown for 5 days at 37 °C with shaking in a chemically defined, nutritionally lean medium. The yeast cells were killed by the addition of 2% formalin. The cells were washed with saline and centrifuged five times at $700 \times g$ for 5 min to remove the formalin. The cells were re-suspended in saline at an approximate concentration of 1×10^7 cells ml^{-1} as determined by the counting chamber method. The rabbits were immunized three times at weekly intervals and then re-challenged on day 49. The rabbits were bled prior to immunization and seven days following re-challenge. Previous determinations indicated that an optimal antibody response was achieved using these parameters. The serum was collected and stored at −20 °C until needed.

Serum specimens

Titrations were performed on the serum specimens obtained from the immunized rabbits and the optimal dilution was determined for use in the antigen detection assay. The rabbit sera were pooled by combining the serum from each rabbit immunized against the pooled *B. dermatitidis* whole yeast cells. The serum specimens were diluted 1:5000 in 0.15% Tween 20 in phosphate buffered saline (NaCl 0.1369 M, KH_2PO_4 0.0015 M, Na_2HPO_4 0.0108, KCl 0.0027 M) (PBS-T, pH 7.4). Sera were also obtained from the same dogs (provided by A.M.L) from which the urine specimens were obtained and used in the indirect ELISA to detect the level of antibody present.

Urine specimens

The urine specimens and the sera from the dogs (provided by A.M.L) were limited by the availability of serial specimens and clinical information about the infected dogs was lacking. The urine specimens were from 12 dogs with known *B. dermatitidis* infections, at various stages of anti-fungal therapy (pre-treatment, 30, 60 days), and from several locations within the endemic area of North America. This provided 36 urine specimens to be tested against the sera from the immunized rabbits. Two urine specimens from dogs with histoplasmosis were also assayed to test for cross reactivity and four urine specimens from uninfected dogs were used as normal controls.

Competitive ELISA method

The horseradish peroxidase competitive binding inhibition ELISA was used for the detection of *B. dermatitidis* antigens in the urine specimens. Microdilution plates (96 well Immunomaxi modified flat bottom high binding, TTP, Switzerland) were coated with 100 μl of T-58 (Tennessee dog isolate) lysate antigen (100 ng ml^{-1}) diluted in carbonate-bicarbonate coating buffer (pH 9.6) and incubated in a humid chamber for 24 h at 4 °C. The plates were washed three times with PBS-T.

Dog urine and 1:5000 serum from rabbits immunized against *B. dermatitidis* killed whole yeast cells (150 μl of each) were combined in microcentrifuge tubes and incubated for 30 min at 37 °C. The complex (100 μl per well) was added to duplicate wells, incubated in a humid chamber for 30 min at 37 °C, and the plates were washed as above. Goat anti-rabbit IgG horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories, KPL, Gaithersburg, MD) at a 1:2000 dilution in PBS-T was added (100 μl per well), incubated for 30 min at 37 °C, and the plates were washed as above. Enzyme substrate (1-step Ultra TMB, Pierce Chemical Company, Rockford, IL) was added (100 μl per well) and incubated for 3 min at room temperature. The reaction was stopped by the addition of 100 μl of 2 N H_2SO_4 to each well. Negative controls containing the known T-58 antigen coated on the plate and the sera from the immunized rabbits were run with the competitive ELISA. Blanks containing substrate and stop solution were used to zero the reader. The absorbance was read at 450 nm using the BIO-RAD model 2550 EIA reader (BIO-RAD, Hercules, CA). Normal urine specimens were used to determine if there was non-specific binding. Absorbance values less than that of the negative control were considered positive for antigen detection.

A standard curve was created by coating the T-58 lysate antigen (100 ng ml^{-1}) onto a microtiter plate and incubated overnight, as described above. A 2-fold dilution, using PBS-T, was performed on the T-58 lysate antigen from an initial protein concentration of 40 μg ml^{-1} to 1.221 ng ml^{-1}. To these dilutions the 1:5000 rabbit sera was added (200 μl of each) and incubated for 30 min. The samples were then added in triplicate to the washed microtiter plate and incubated in a humid chamber for 30 min. The plate was then washed and 1:2000 goat anti-rabbit IgG horseradish peroxidase conjugate (KPL) was added. The plate was incubated, rinsed, and 1-step Ultra TMB substrate (Pierce) was added. After reacting at room temperature for 3 min 100 μl of 2 N H_2SO_4 was added to stop the reaction and the absorbance was read at 450 nm using the BIO-RAD EIA reader.

Indirect ELISA method

The microdilution plates were coated with the T-58 lysate antigen and incubated overnight, as described above. Upon rinsing the plates three times with PBS-T, the dog sera (1:5000) were added in duplicate and incubated in a humid chamber for 30 min at 37 °C. The plates were washed as above. Goat anti-dog IgG horseradish peroxidase conjugate (KPL) at a 1:2000 dilution in PBS-T was added (100 μl per well) incubated for 30 min at 37 °C, and the plates were washed as above. One-step Ultra TMB substrate (Pierce) was added (100 μl per well) and incubated for 3 min at room temperature. The reaction was stopped by the addition of 100 μl of 2 N H_2SO_4 to each well. Blanks containing substrate and stop solution were used to zero the reader. The absorbance was read at 450 nm using the BIO-RAD EIA reader.

Results

Antibodies produced by rabbits immunized with killed *B. dermatitidis* whole yeast cells were effective at detecting *B. dermatitidis* antigens in dogs with blastomycosis (Figure 1) with 100% detection observed for all 36 urine specimens from dogs with known *B. dermatitidis* infections. Histoplasmosis cross reactivity was observed in one of the two urine specimens from dogs infected with *H. capsulatum*. One of the four normal urine specimens exhibited competitive binding as would be expected with a positive sample.

The standard curve for the T-58 lysate antigen (Figure 2) shows that for the 40 to 5 μg ml^{-1} range the concentration of antigen was too great leaving no antibody to bind to the competitive inhibition plate. However, a curve is apparent from 2.5 μg ml^{-1} to 9.776 ng ml^{-1}, which is the endpoint as that is also the absorbance value observed for the negative control.

The indirect ELISA was used to determine the level of *B. dermatitidis* antibody present in the dogs. Antibody was detected in 100% of the serial specimens. The level of antibody detection varied among the dogs. Differing levels of antibodies were observed with the progression of anti-fungal therapy (Figure 3) as discussed below.

Discussion

This initial study has shown that *B. dermatitidis* antigens are detectable in urine specimens when

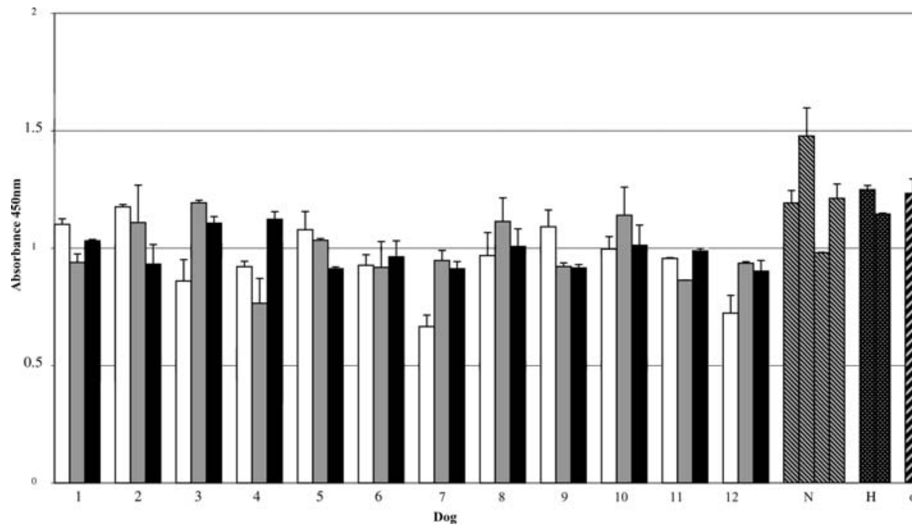

Figure 1. The competitive inhibition ELISA absorbance values from the urine specimens of 12 dogs infected with blastomycosis are shown at pre-treatment, 30 and 60 days of anti-fungal therapy, as well as four (N) normal urine specimens, two (H) *Histoplasma capsulatum* urine specimens and the (C) negative control. All values less than the negative control are considered positive for antigen detection. Error bars indicate the standard deviation of the specimens.

using the competitive ELISA method with antibody from rabbits immunized with *B. dermatitidis* killed whole yeast cells (Figure 1). There did not seem to be any specific time frame with regard to the optimal amount of antigen associated with the specific time interval that the urine specimens were collected. The greatest amount of antigen was evidenced in five specimens collected prior to therapy, four specimens collected on day 30 and three specimens collected at the 60 day interval following treatment.

The sera from the immunized rabbits detected *B. dermatitidis* in a sensitive manner, but the degree of specificity was lower. A known amount of *B. dermatitidis* killed whole yeast cells was administered to the rabbits at specified time intervals and the level of antibody production in the rabbits was monitored with an indirect ELISA.

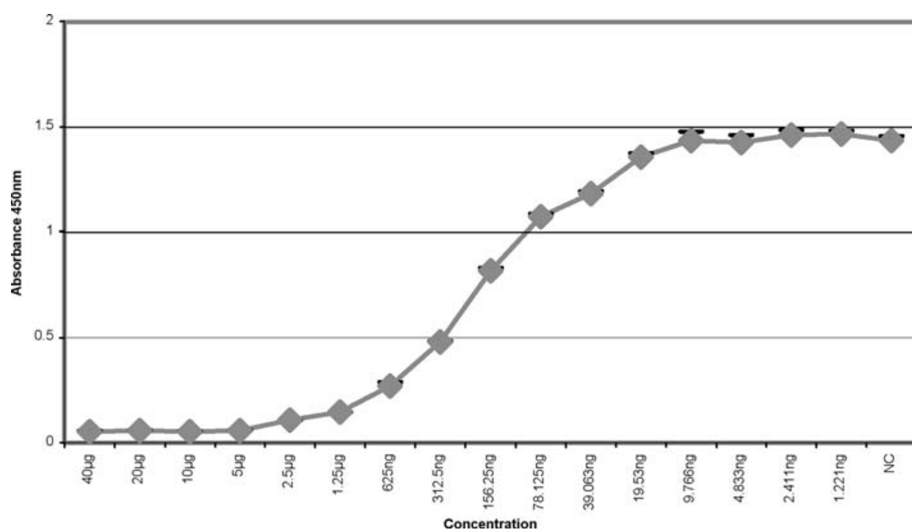

Figure 2. The competitive inhibition ELISA standard curve of the T-58 lysate antigen with concentrations ranging from 40 μg ml^{-1} to 1.221 ng ml^{-1} and the negative control (NC) are shown. Error bars indicate the standard deviation of the specimens.

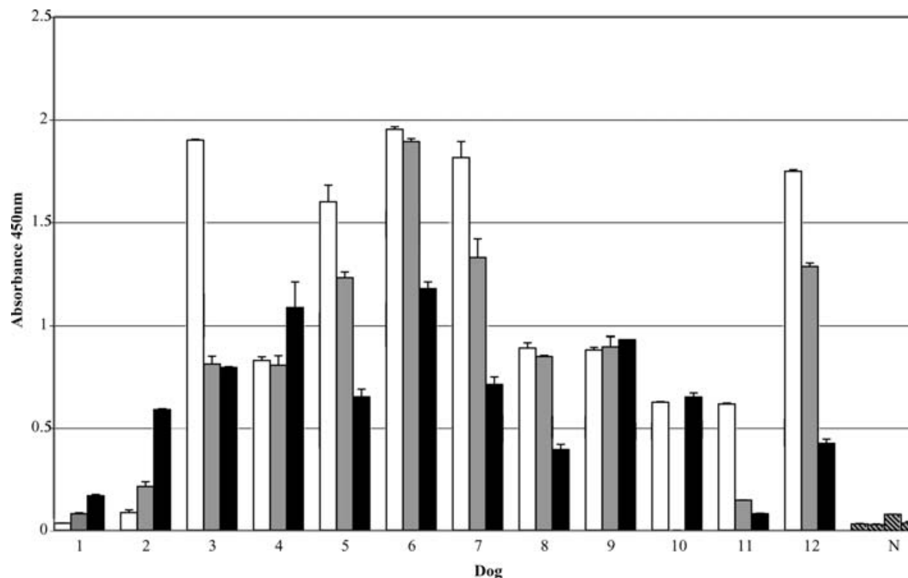

Figure 3. The indirect ELISA absorbance values from the sera of 12 dogs infected with blastomycosis are shown at pre-treatment, 30 and 60 days of anti-fungal therapy, as well as four (N) normal sera specimens. Error bars indicate the standard deviation of the specimens. Note that there is not a 30 day sample for dog 10.

The rabbit sera used in this study was collected seven days after a booster immunization, which elicited a secondary response in the rabbits.

The indirect ELISA (Figure 3) was able to detect *B. dermatitidis* antibody in the sera from the infected dogs. Antibody levels in the sera are expected to vary from dog to dog. In contrast to the antigen detection assay, the optimal amount of antibody detection was evidenced in seven specimens collected prior to therapy and five specimens collected at the 60 day interval following treatment. Seven dogs did show a decline in antibody detection at the 60 day interval. The stage or duration of the infection were parameters that were not defined when the diagnosis was made.

The ability to detect antigens in urine specimens may aid in the diagnosis of blastomycosis in immunocompromised individuals and could also mean an earlier, non-invasive method for detection in dogs. By detecting antigens produced by the fungal organism instead of antibodies produced by the infected individual, a more rapid diagnosis may be made. Since the test is not dependent upon antibody levels it could also be beneficial for monitoring the progress of anti-fungal therapy. Further research is in progress to increase the specificity and the optimal parameters of the competitive binding inhibition ELISA.

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Address for correspondence: Dr. Gene M. Scalarone, Department of Biological Sciences, Idaho State University, Pocatello, Idaho, 83209, USA
Phone: 208-282-3374; Fax: 208-282-4570
E-mail: scalgene@isu.edu